E. coli cell-free system supplemented with *E. coli* cell membrane fractions, heme A and Cu_2SO_4 (about 500 μg of the proteins/ml was produced). Blue native PAGE and SDS-PAGE of dodecylmaltoside-solubilized ^{35}S-labeled proteins revealed a protein complex with a normal subunit stoichiometry. Synthesized proteins were purified by a MonoQ column chromatography, giving a fraction exhibiting the KCN sensitive ferrocytochrome *c* oxidation activity and the normal Soret/visible spectra. The elution volume of the fraction identical to that of the authentic enzyme also suggests the native conformation of the synthesized enzyme. These results indicate successful cell-free synthesis of the native enzyme. However, the amount of the functional enzyme was still about 1% of the synthesized proteins.

doi:10.1016/j.bbabio.2008.05.278

S11.24 Mutations of possible proton-transfer pathways of bovine heart cytochrome *c* oxidase

Kunitoshi Shimokata[a], Yukie Katayama[a], Makoto Suematsu[a], Tomitake Tsukihara[b], Shinya Yoshikawa[c], Hideo Shimada[a]
[a]*Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, Japan*
[b]*Institute of Protein Research, Osaka University, Japan*
[c]*Graduate School of Life Science, University of Hyogo, Japan*
E-mail: shimada@sc.itc.keio.ac.jp

X-ray structures of the bovine enzyme at 1.8–1.9 Å resolution show a possible proton-pumping pathway (H-pathway) composed of a hydrogen bond network and a water channel. The former includes the peptide bond, which could facilitate unidirectional proton transfer. Mutant enzymes in which proline is introduced to block proton transfer through the peptide bond and in which bulkier amino acids are introduced to the water channel to block its water-transfer function showed complete abolishment of the proton pumping without affecting the dioxygen reduction activity. These results and the previous proton pumping site mutation (Asp51Asn) result strongly support the proposed proton pumping function of H-pathway. On the other hand, a mutation (Asn98Asp in bovine numbering) of bacterial enzymes in D-pathway, a possible proton-transfer pathway connecting the negative-side surface with the dioxygen reduction site, abolishes proton pumping without impairing the O_2 reduction activity, suggesting that D-pathway of bacterial enzymes convey pumping-protons also. However, the Asn98Asp mutant of the bovine D-pathway negligibly influenced the O_2 reduction and proton pumping (H^+/e^-, 0.64 (n=8) for wild type and 0.65 (n=7) for the mutant). The present results suggest that function of D-pathway is not conserved between bovine and bacterial enzymes.

doi:10.1016/j.bbabio.2008.05.279

S11.25 X-ray structure of carbon monoxide at copper site of the dinuclear site of cytochrome *c* oxidase

Kazumasa Muramoto[a], Naoki Nakagawa[a], Maki Taniguchi[a], Katsumasa Kanda[a], Kyoko Shinzawa-Itoh[a], Tomoko Maeda[a], Eiki Yamashita[b], Tomitake Tsukihara[b], Shinya Yoshikawa[a]
[a]*Department of Life Science, University of Hyogo, Japan*
[b]*Institute for Protein Research, Osaka University, Japan*
E-mail: yoshi@sci.u-hyogo.ac.jp

The copper site (Cu_B) in the O_2 reduction site of cytochrome *c* oxidase is silent to most of spectroscopic techniques and thus the role of Cu_B in the O_2 reduction mechanism is poorly understood. The fully reduced carbon monoxide (CO) derivative of bovine heart cytochrome *c* oxidase photolyzed below 140 K shows C–O stretch band at 2062 cm^{-1}, assignable to CO bound at Cu_B. However, the infrared result provides no direct geometric information for the bound CO. Electron density map of the fully reduced-CO bound form of bovine heart cytochrome *c* oxidase under light conditions at 100 K at 1.8 Å resolution shows an electron density peak assignable to CO near Cu_B atom. The F_o–F_c map strongly suggests a side-on binding of CO to Cu_B, although the possibility of an end-on binding cannot be excluded at this resolution. The distances between Cu_B and the two atoms of CO are 2.5 Å and 2.4 Å, suggesting a fairly weak metal/ligand interaction. The weak interaction is likely to contribute the stability of the oxygenated form of the enzyme (Fe_{a3}–O_2), which is prerequisite for the four electron reduction of O_2 at Fe_{a3}.

doi:10.1016/j.bbabio.2008.05.280

S11.26 Structural analyses for lipid/protein interactions in bovine heart cytochrome *c* oxidase

Kyoko Shinzawa-Itoh[a], Hiroshi Aoyama[b], Kazumasa Muramoto[a], Hirohito Terada[a], Tsuyoshi Kurauchi[a], Yoshiki Tadehara[a], Akiko Yamasaki[c], Takashi Sugimura[c], Sadamu Kurono[d], Kazuo Tsujimoto[d], Tsunehiro Mizushima[e], Eiki Yamashita[e], Tomitake Tsukihara[e], Shinya Yoshikawa[a]
[a]*Department of Life Science, University of Hyogo, Japan*
[b]*RIKEN Harima Institute, Japan*
[c]*Department of Material Science, University of Hyogo, Japan*
[d]*Graduate School of Material Science, Japan Advanced Institute of Science and Technology, Japan*
[e]*Institute for Protein research, Osaka University, Japan*
E-mail: yoshi@sci.u-hyogo.ac.jp

All 13 lipids, including two cardiolipins, one phosphatidylcholine, three phosphatidylethanolamines, four phosphatidylglycerols and three triglycerides were identified in a crystalline bovine heart cytochrome *c* oxidase (C*c*O) preparation. The chain lengths and unsaturated bond positions of the fatty acid moieties determined by mass spectrometry suggest that each lipid head group identifies its specific binding site within C*c*O. Binding of dicyclohexylcarbodiimide to the O_2-transfer pathway of C*c*O causes two palmitate tails of phosphatidylglycerols to block the pathway, suggesting that the palmitates control the O_2 transfer. The phosphatidylglycerol with vaccenate (*cis*-Δ^{11}-octadecenoate) was found in C*c*O of *Paracoccus denitrificans*, a possible ancestor of mitochondrion. This indicates that the vaccenate is conserved in bovine C*c*O in spite of the abundance of oleate (*cis*-Δ^9-octadecenoate). The X-ray structure indicates that the protein moiety selects *cis*-vaccenate against *trans*-vaccenate for the O_2-transfer pathway. These results suggest that vaccenate plays a critical role in the O_2-transfer mechanism and that the lipid binding specificity is determined by both the head group and the fatty acid tail.

doi:10.1016/j.bbabio.2008.05.281

S11.27 X-ray structural analysis of zinc/cadmium inhibitory site in bovine heart cytochrome *c* oxidase

Kazumasa Muramoto[a], Kazunori Maeda[a], Takeshi Takahashi[a], Kunio Hirata[b], Kyoko Shinzawa-Itoh[a], Tomoko Maeda[a], Shinji Yoko-o[a], Eiki Yamashita[b], Hiroshi Aoyama[c], Tomitake Tsukihara[b], Shinya Yoshikawa[a]
[a]*Department of Life Science, University of Hyogo, Japan*
[b]*Institute for Protein Research, Osaka University, Japan*
[c]*RIKEN Harima Institute, Japan*
E-mail: muramoto@sci.u-hyogo.ac.jp

Cytochrome *c* oxidase (CcO) catalyzes the oxygen reduction coupled to the electron and proton transfer. Previous biochemical studies indicated that Zinc (Zn) inhibited proton transfer by binding either inside or outside surface of CcO. Similar inhibition is also caused by (Cadmium) Cd. To identify the Zn/Cd inhibitory sites, we have carried out the X-ray structural analyses of bovine heart CcO–Zn/Cd complex. We found several Zn/Cd-binding sites by using the crystal of dimeric CcO. The highest affinity Zn/Cd-binding site (Zn2/Cd1 site) is located at the inside surface of the subunit III. The second highest affinity site (Zn3/Cd2 site) on the inside surface is located at the D-pathway entrance. The zinc binding affinity for the second site suggests that the zinc site is tightly coupled with the proton-pumping site. Recently, we analyzed Zn/Cd-binding to monomeric CcO which gives crystal packing different from that in the dimeric CcO crystal. The X-ray structural analysis showed Zn-binding to the Zn2, Zn3 and additional sites including the site near the K-pathway entrance. Several Zn-binding sites have been found on the outside surface. However none of them is located on the subunit I surface from which pumping protons exit.

doi:10.1016/j.bbabio.2008.05.282

S11.28 The finding of the CBB_3-oxidase gene and the evidence for enzyme expression in extremely alkaliphilic bacteria

Denis A. Morozov, Maria S. Muntyan, Sergey S. Klishin
A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Russia
E-mail: denmorru@mail.ru

The aim of this study was to determine whether the *cbb*$_3$-oxidase is expressed in new representatives of extremely alkaliphilic bacteria *Thioalkalivibrio* with the optimum of growth at pH=10. For this purpose we used the synthetic oligopeptides identical to the C-terminal parts of the catalytic subunit and then worked out the specific antibodies against the catalytic subunit. A *cbb*$_3$-type oxidase was shown to be expressed in membranes of extremely alkaliphilic bacterium *Thioalkalivibrio versutus* using polyclonal rabbit anti-ccoN antibodies. The expressed oxidase is composed of 48, 34 and 29 kDa subunits, the two smaller of them being presented by cytochromes *c*. The 48 kDa subunit cross reacting with anti-ccoN antibodies was detected as a catalytic one. Sequence of the 5′-end terminal fragment of the catalytic subunit gene produced significant alignment with sequences of *Methylobacillus flagellatus* KT and *Thiobacillus denitrificans cbb*$_3$-type oxidase *ccoN*, displaying only distant phylogenetic relationship to them. Acknowledgments: authors thanks N. Pozdnyakova for assistance in work with animals.

doi:10.1016/j.bbabio.2008.05.283

S11.29 Possible proton transfer mechanism through peptide groups in the H-pathway of the bovine cytochrome *c* oxidase

Katsumasa Kamiya[a], Mauro Boero[b,c,d], Masaru Tateno[b,c,d], Kenji Shiraishi[b,c,d], Atsushi Oshiyama[b,d,e]
[a]*Institute for Picobiology, Graduate School of Life Science, University of Hyogo, Japan*
[b]*Center for Computational Sciences, University of Tsukuba, Japan*
[c]*Graduate School of Pure and Applied Sciences, University of Tsukuba, Japan*
[d]*CREST, Japan Science and Technology Agency, Japan*
[e]*School of Engineering, The University of Tokyo, Japan*
E-mail: kkamiya@sci.u-hyogo.ac.jp

A recently proposed proton transfer path (H-path) in bovine cytochrome *c* oxidase involves the peptide group connecting Tyr440 and Ser441 and interrupts the continuous hydrogen-bond network across which protons are expected to propagate. Our first-principles calculations show that the porpagation is not hindered, but occurs via a multi-step process. A proton is initially transferred to the carbonyl oxygen of a keto form of the Tyr440-Ser441 peptide group [–CO–NH–], producing an imidic acid [–C(OH)–NH–] as a metastable state. The amide proton of the imidic acid is then transferred in a barrierless way to the deprotonated carboxyl group of the Asp51 side chain, leading to the formation of an enol form [–C(OH)=N–]. Eventually, an enol-to-keto tautomerization is realized via a double proton transfer in the two adjacent Tyr440-Ser441 and Ser441-Asp442 peptide groups. An analysis of the pathway shows that each elementary process occurs through the shortest distance, thus preserving the X-ray structure, and the path is characterized by a reasonable activation barrier.

doi:10.1016/j.bbabio.2008.05.284

S11.30 Oxygen reaction in the *cbb*$_3$-type cytochrome *c* oxidase from *Rhodobacter Sphaeroides*

Virve Rauhamäki, Michael I. Verkhovsky, Mårten Wikström
Helsinki Bioenergetics Group, Institute of Biotechnology, University of Helsinki, Finland
E-mail: virve.rauhamaki@helsinki.fi

The *cbb*$_3$-type cytochrome *c* oxidase is a proton-pumping terminal oxidase present solely in bacteria. The enzyme is expressed mainly under very low oxygen tension and it has been shown to have a high apparent affinity for oxygen. Therefore it is of interest to investigate the properties of the enzyme that allow it to function under such conditions. In this work we have studied directly the reaction of cytochrome *cbb*$_3$ with molecular oxygen using fast kinetic approaches. The flow-flash method, where a reduced carbon monoxide inhibited enzyme is mixed with oxygenated buffer and the reaction is started by a laser flash, allowed us to follow the optical changes during the catalytic reaction at different oxygen concentrations as well as the potential generation across the membrane that takes place as a result of the charge transfer. The initial reaction with oxygen was found to be relatively slow ($k_{on} \sim 2\times10^7$ 10^7 M^{-1} s^{-1}) and the consequent steps of reduction were coupled to potential generation. Therefore the efficiency in reducing molecular oxygen at low oxygen concentration is not based on a particularly fast binding of O_2, but rather on the irreversibility of the oxygen binding to the enzyme.

doi:10.1016/j.bbabio.2008.05.285

S11.31 Nitric oxide reductase from *Paracoccus Denitrificans* —what are the five conserved glutamates in NorB good for?

Ulrika Flock, Peter Lachmann, Pia Ädelroth
Department of Biochemistry and Biophysics, Stockholm University, Sweden
E-mail: Ulrika.Flock@dbb.su.se

The *c*-type nitric oxide reductase (NOR) from *Paracoccus denitrificans* is an odd member of the heme copper oxidase superfamily. It catalyses NO reduction; $2NO+2e^-+2H^+ \rightarrow N_2O$ N_2O+H_2O and also oxygen reduction as a side reaction. All known *c*-type NORs have been shown to have five conserved glutamates (E) in the catalytic subunit (here NorB). These are, by *Paracoccus denitrificans* numbering, the E122, E125, E198, E202 and E267. The E122 and E125 are presumed to face the periplasm and the E198, E202 and E267 are thought to be located in the interior of the membrane, not far from the catalytic site.